PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | HILL | (11) International Publication Number: WO 91/15571 |
|---|--------------------|--|
| (51) International Patent Classificati n ⁵ : | A1 | (II) Intolinational I assessed to the last of the last |
| C12N 1/19, 15/81, 15/31 // (C12N 1/19, C12R 1/84) | | (43) International Publication Date: 17 Oct ber 1991 (17.10.91) |
| (21) International Application Number: PCT/GE (22) International Filing Date: 28 March 1991 | | patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European |
| (30) Priority data: 9007416.2 2 April 1990 (02.04.90) | | patent), NL (European patent), NO, SE (European patent), US. |
| (71) Applicant (for all designated States except US): TH COME FOUNDATION LIMITED [GB/GB] House, 160 Euston Road, London NW1 2BP (| ; Unic | L- Published With international search report. |
| (72) Inventors; and (75) Inventors/Applicants (for US only): CLARE, Jeff [GB/GB]; ROMANOS, Michael, Anthony Langley Court, Beckenham, Kent BR3 3BS (G | US/G | hn 3]; |
| (74) Agent: STOTT, M., J.; The Wellcome Foundation Langley Court, Beckenham, Kent BR3 3BS (G | n Limit B). | ed, |
| | | |
| - | | · |
| • | | |
| (54) Title: EXPRESSION OF HETEROLOGOUS F | ROTE | IN IN YEAST |
| (57) Abstract | | |
| Production of <i>Bordetella</i> pertactin antigens by a DNA encoding the antigens and <i>Pichia</i> transformantigen. | express s conta | on in the methyltrophic yeast, <i>Pichia</i> ; expression vectors containing ning the one of more copies of the DNA encoding a pertactin anti- |
| | | |
| | | • |
| | | |
| | | |
| , | | • |
| · | | |
| | | • |
| | | |
| | | |
| | | |
| | | • |
| | | |
| · · | | |

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| AT | Austria | ES | Spain | MG | Madagascar |
|-----|--------------------------|----|------------------------------|-----|--------------------------|
| AU | Australia | PI | Finland | ML. | Mali |
| BB | Barbados | FR | France | MN | Mongolia |
| BB | Belgium | GA | Gabon | MR | Mauritania |
| BF | Burkina Faso | GB | United Kingdom | MW | Malawi |
| BG | Bulgaria | GN | Guinca | NL. | Netherlands |
| BJ | Benin | GR | Greece | NO | Norway |
| BR | Brazil . | HU | Hungary | PL | Poland |
| CA | Canada | IT | Italy | RO | Romania |
| CF | Central African Republic | 4£ | Japan | SD | Sudan |
| CC | Congo | KP | Democratic People's Republic | SB | Sweden |
| CH | Switzerland | | of Korea | SN | Senegal |
| а | Côte d'Ivoire | KR | Republic of Korea | SU | Soviet Union |
| СМ | Cameroon | LI | Liechtenstein | TD | Chad |
| CS | Czechoslovakia | LK | Sri Lanka | TG | Togo |
| DE. | Germany | LU | Luxembourg | US | United States of America |
| DK | Denmark | MC | Monago | 03 | Cined Seeds Of Afficiles |

EXPRESSION OF HETEROLOGOUS PROTEIN IN YEAST

The present invention relates to the expression of heterologous protein in yeast, more particularly to the production of <u>Bordetella</u> pertactin antigens in <u>Pichia</u>, novel expression vectors containing the DNA sequences, and <u>Pichia</u> strains transformed therewith.

Bordetella pertussis causes whooping cough, an acute respiratory disease which is serious and debilitating in humans, children being particularly susceptible. The organism is responsible for approximately 1-million deaths each year, although this is being to some extent controlled in the developed countries by large scale immunisation programmes. It has been found that immunisation against B.pertussis is very effective at preventing the disease, and that failure to vaccinate does lead to increased incidence of the disease. In practically all areas, immunisation is effected using a whole cell B.pertussis vaccine which has been found to be relatively effective in preventing the disease and infant mortality.

However, public acceptance of whole cell vaccines has decreased due to side-effects and controversy over rare neurological complications attributed to such vaccine preparations. Consequently, researchers have been looking for safer, effective, acellular vaccines consisting of purified <u>Bordetella</u> antigens.

Surface antigen of <u>B.pertussis</u> is known to elicit a humoral and a cellular immune response in humans. It is disclosed as ACAP in European Patent Application published under No. 162639 and is now known as P.69 (I.G. Charles <u>et al.</u> Proc.Natl.Acad.Sci. USA, vol. <u>80</u>, 3554-3558 (1989)). It is likely to be an important component of any future acellular vaccine against <u>B.pertussis</u> infections.

<u>B.parapertussis</u> and <u>B.bronchiseptica</u> are closely related to the <u>B.pertussis</u> organism. <u>B.parapertussis</u> is also responsibl for utbreaks of whooping c ugh in man (Zeuler <u>et al</u>. J.p diatr. 9:493-497 (1946); <u>B.bronchiseptica</u> is known to cause respiratory diseases in animals, particularly atrophic rhinitis in pigs (Harris and Switzer Am.J. Vet. Res. <u>29</u> 777-785 (1968)).

B.parapertussis and B.bronchiseptica appear to present antigens related to B.pertussis P.69, with molecular masses of 70 and 68kDa respectively. These Bordetella antigens, which are referred to hereinafter as 'pertactin antigens', are known to bind to the BB05 antibody but appear to have different immunogenic properties (I.G. Charles et al. Proc. Natl. Acad. Sci. USA Vol. 80 3554-3558 (1989)). Only small amounts-of-pertactin antigen can be isolated from cultures of Bordetella organism. It is preferable for the production of antigens on a commercial scale, to be able to produce large quantities.

E.coli is known as a host organism for the manufacture of heterologous proteins such as antigens, in quantity, but has certain drawbacks since it contains toxic pyrogenic factors (lipopolysaccharides from the cell wall) which must be rigorously excluded from the final product. The ease with which these factors may be excluded will depend on the method of purification. However, it would be advantageous to eliminate the possibility of contamination altogether simply by using a non-toxic organism as the host, such as yeast.

When baker's yeast, <u>Saccharomyces cerevisiae</u>, is used as the host organism, poor expression levels of heterologous protein are frequently obtained. (Kingsman, <u>et al.</u>, Biotechnology & Genet. Engin. Reviews. Vol. <u>3</u> 377-416, 1985). Use of the yeast <u>Pichia pastoris</u> as a host for the expression of heterologous protein is also known (European Patent Publication Nos. 0180899 and 0263311). However, expression of membrane proteins in yeast is generally problematic since these proteins can interact with yeast cell membranes causing toxic effects to the yeast cell and reduced product yields. Examples of such difficulties have been described and include the expression of p ly ma virus middl -T antigen (Belsham, <u>et al</u>. Eur. J. Biochem. <u>156</u>

413-421, 1986); expressi n f the bacterial membrane protein OmpA (Janowitz, et al. Gene 20, 347-358, 1982); and expression of influenza virus haemagglutinin (Jabbar, et al., Proc. Natl. Acad. Sci. USA 82, 2019-2023, 1985).

The present inventors have found a means of producing good levels of expression of the <u>Bordetella</u> pertactin antigens by culturing <u>Pichia</u> transformants containing at least one copy of the DNA encoding a pertactin antigen or an <u>antigenic</u> fragment thereof.

Accordingly, in a first aspect of the invention there is provided a <u>Pichia</u> microorganism transformed with DNA for the expression of a pertactin antigen whose amino acid sequence is at least 95% homologous with that set forth in Figures 1A, 1B or 1C (SEQ ID NO: 1,2 or 3), or an antigenic fragment thereof.

It is preferable if the amino acid sequence described above is at least 98% homologous with that set forth in Figures 1A, 1B or 1C (SEQ ID NO: 1, 2 or 3) or an antigenic fragment thereof.

A pertactin antigen from B.pertussis includes the antigen whose amino acid sequence is at least 95% homologous with, but is preferably substantially the same as, that set out in Figure 1A (SEQ ID NO: 1). This antigen is denoted P.69. Α antigen pertactin B. bronchiseptica includes the antigen whose amino acid sequence is at least 95% homologous with, but is preferably substantially the same as, that set out in Figure 1B (SEQ ID NO: 2). This antigen is denoted P.68. A pertactin antigen from B.parapertussis includes the antigen whose amino acid sequence at least 95% homologous with, but is preferably substantially the same as, that set out in Figure 1C (SEQ ID NO: 3). This antigen is denoted P.70.

The DNA for the expression of a pertactin antigen may encode a larger precursor which has a molecular w ight of approximately 94kD and which is pr cessed within the cell to the desired antigen. In the case of

P.69 the precursor is appr ximately 93.5kD. The DNA enc ding it has been cloned and sequenced by Charles et al., (PNAS, 86, pp 3554-3558, (1989)). The precursor of the P.68 antigen of <u>B.bronchiseptica</u> is approximately 94kD and the precursor for P.70 of <u>B.parapertussis</u> is approximately 95kD.

<u>Pichia</u> microorganisms transformed with DNA for the expression of an antigenic fragment of a pertactin antigen are also encompassed by the invention. The fragments preferably contain no more than 50 amino acid residues. More preferably they contain between 5 and 25 residues. The fragments most preferably comprise a defined antigenically effective sequence which essentially consists of amino acid residues 547 to 552 of the P.69 protein of <u>B.pertussis</u>.

This sequence is : PGPQPP (SEQ ID NO: 4)

The corresponding sequence for other strains of <u>B.pertussis</u> and for strains of <u>B.parapertussis</u> and <u>B.bronchiseptica</u> can be readily determined by lining up the amino acid sequence of the P.69 antigen, the P.70 antigen or the P.68 antigen respectively with the P.69 sequence shown by Charles <u>et al</u> (1989) referred to hereinbefore.

The fragments described above also include a sequence which essentially consists of the amino acid residues 544 to 566 of the P.69 protein of <u>B.pertussis</u>.

This sequence is : APQPGPQPPQPPQPPQPPQPEAPAPQP (SEQ ID NO: 5)

This sequence and the corresponding sequence for the P.70 antigen of <u>B.parapertussis</u> and the P.68 antigen of <u>B.bronchiseptica</u> can be aligned. A further fragment of interest is a 60 kD fragment encoded by the C terminal end of the DNA for P.69 which has been identified by Charles <u>et</u>. <u>al</u>. (1989) referred to hereinbefore as encoding an antigenic fragment f P.69.

Transf rmation of the organism may be carri d out by any known method in the literature (Beggs, Nature 275, 104-109 (1978)). preferable to use the sphaeroplast method described by Cregg et al., Bio/Technology 5 479-485 (1987). The Pichia organism is preferably transformed with an expression cassette. Expression cassettes include DNA sequences in addition to that encoding the sequence of interest, in this instance the DNA encoding a pertactin antigen, such as and translational transcriptional initiation and termination sequences. The cassette may also include regulatory (i.e. promoter) and/or selectable marker sequences. Such expression cassettes are well known in the art and it is well within the ability of the skilled man to construct them. The expression cassette may form part of a vector construct or a naturally-occurring plasmid.

In a preferred embodiment the vector construct used to transform Pichia cells contains the promoter from the methanol-inducible AOX1 gene to drive expression of the DNA encoding a pertactin antigen. In particular, the present invention provides the vector pPIC3-60.5K. This vector, digested with BglII, will integrate in the host chromosomal AOX1 locus. The resultant aox1 transformants have the Mut^S (methanol-utilisation slow) phenotype and can therefore be selected.

In a preferred aspect of the invention there is provided a <u>Pichia</u> organism transformed with more than one copy of the DNA encoding a pertactin antigen or an antigenic fragment thereof preferably integrated in the <u>Pichia</u> chromosomal DNA. It is preferable for the organism to contain greater than 5 copies preferably greater than 10 copies and most preferably between 5 and 30 copies of the DNA encoding pertactin antigen or an antigenic fragment thereof.

Such transformants can produce up to 5% of cell protein as the desired protein. In optimal fermenter conditions, levels of up to 10% of cell protein can be produced as the heterologous antigen. At such levels the majority of the antigon is insoluble. This is advantageous since

the material can be readily isolated, r natur d, and purified from the insoluble fraction.

The preferred Pichia organism referred to above is Pichia pastoris.

The invention further provides a process for producing a pertactin antigen which comprises culturing a transformed <u>Pichia</u> microorganism of the present invention.

Culturing of the transformed microorganism is carried out by known methods in for example a yeast extract band medium using a shake flask. For optimal conditions it is preferable to use a fermenter equipped for monitoring pH, 0_2 , stir speed, temperature and or air flow, to control the cells' environment. The antigen produced by such methods can be isolated by centrifugation and purified by chromatography.

In order to obtain organisms containing more than one copy of the DNA encoding a pertactin antigen it is necessary to prepare a library of transformants using standard techniques for example as described by Cregg et al., Bio/Technology 5: 479-485 (1987). Transformants containing at least one integrated copy of the DNA encoding a pertactin antigen form approximately 10-20% of these transformants. Those containing multiple copies of the DNA are then identified by screening. Individual transformants are grown on microtitre plates, each microculture is then transferred onto nitrocellulose filters and probed by Southern Hybridisation using pertactin-specific radiolabelled to high specific activity using random-primed labelling (Feinberg et al., (1989) Anal. Biochem., 132- 6-13). A weak signal indicates that transformants contain a single copy of the DNA, a stronger signal indicates that the transformant contains more than one copy of DNA. The filters can then be mapped with the microtitre plates to identify the desired multi-copy microculture(s).

The present invention therefor provides a process of producing a Pichia organism according to the invention comprising:

- transforming a <u>Pichia</u> organism with a vector contruct containing DNA encoding a pertactin antigen or a fragment thereof and a selectable marker.
- ii) screening <u>Pichia</u> cells by means of selectable marker to select a transformed organism.
- iii) screening said transformed organism to identify transformants containing said DNA or fragment thereof.

Another aspect of the invention provides a process for the enhanced production of pertactin antigen comprising:

- a) transforming a <u>Pichia</u> organism with multiple vectors constructs containing DNA encoding a pertactin antigen or a fragment thereof operably linked to a promoter to drive expression of said DNA; and thereafter
- b) culturing the resultant transformed organism under suitable conditions to obtain production of the antigen encoded by the DNA.

The present invention will now be exemplified further with reference to the accompanying figures.

FIGURE LEGENDS

Fig.1A. The amino acid sequence of P.69 and the DNA sequence encoding P.69 from <u>B.pertussis</u> and its 93.5 kD precursor.

Fig.18. The amino acid s quence of P.68 and the DNA sequence enc ding P.68 from <u>B.bronchiseptica</u> and its 94 kD precursor.

Fig.1C. The amino acid sequence of P.70 and the DNA sequence encoding P.70 from B.bronchiseptica and its 95 kD precursor.

Fig.2A. Construction of the vector pPIC3-60.5K.

Fig. 2B A plasmid map of the vector pPIC3-60.5K is shown in part A. Part B gives details of the DNA sequence of the DNA encoding P.69 that was used in the construction of pPIC3-60.5K. The EcoRI-NheI DNA fragment used contains the region from AvaI (nt.315) to BgII (nt.1979) from the 93kD precursor gene (Charles et al. (1989), PNAS 86, 3554-3558), flanked by sequences derived from the vector pPERtac8 (Makoff et al. Bio/Technology 8,1030 (1990)). This fragment was inserted into pPic2 using the adapter oligonucleotides shown, which encode the 5' end of the DNA encoding P.69 gene.

Fig. 3 DNA dot blot screen of GS115/pPIC3-60.5K Mut^S transformants. The filter shown had 56 transformants, a positive control (position E10: multi-copy transformant SL 22, identified in a previous screen), and a negative control (E11: GS115). Most of the transformants in the screen gave a weak signal of similar intensity, a very small proportion gave a much stronger signal indicative of multi-copy integration (A3, A4, B6: designated nos. SL3, SL4 and SL18). Four further filters were screened in the same way, but no more multi-copy transformants were found.

Fig. 4 Western blot analysis of P.69 pertactin induced <u>P.pastoris</u> cell extracts. <u>P.pastoris</u> transformants were induced for two days as described in Example 4. Proteins were separated by electrophoresis in 7.5% SDS-polyacrylamide gels and detected in Western blots using monoclonal antibody BB05 (Bio/Technology <u>8</u>, 1030 (1990)). Tracks contained; authentic <u>B.pertussis</u> pertactin (1); or cell extracts from shake-flask induced; <u>P.pastoris</u> single-copy integrant SL1 (2), or from multi-copy integrant SL3 (3), SL4 (4), SL18 (5), and SL22 (6). Expressi n levels were estimated by comparison with known amounts of pertactin:

Fig. 5 Analysis of pertactin expression from a SL22 ferment r induction. A fermenter protocol of batch growth in glycerol, followed by glycerol-starvation, then a controlled methanol feed was used as described in Example 5. Protein extracts (50µg) prepared from samples taken at different times after induction were analysed: tracks 2 to 9 correspond to -1, 0, 2, 4, 7, 23, 30, 50 hrs, respectively. Track 1 contained protein markers (Amersham Rainbow markers: myosin 200kDa, phosphorylase b 92.5kDa, bovine serum albumin 69kDa, ovalbumin 46kDa, carbonic anhydrase 30kDa). The induced pertactin band is indicated by an arrow. Densitometric scanning of the stained gel (Joyce-Loebl, Chromoscan) indicated that pertactin reached approximately 10% of cell protein after 30 hrs.

SDS-polyacrylamide gel analysis of pertactin purification. Induced SI4 cells were harvested, suspended in buffer A (50mM tris HCl pH8.0, 0.1M NaCl) + 1% triton X-100, and lysed with glass beads using a Bead Beater (Biospec Products, Bartesville, OK). Insoluble protein was pelletted by centrifugation, washed and suspended in guanidinium thiocyanate/buffer A. On dialysis pertactin remained soluble while yeast proteins could be removed by centrifugation. Solubilised pertactin was loaded onto a chelating Sepharose column charged with zinc and equilibrated with buffer A. After washing with 0.5M NaCl, pertactin was eluted in 50mM MES pH6.0, 0.1M NaCl. Pertactin was further purified by chromatography on Q-sepharose in 50mM tris HCl pH8.0, with elution using a 0 to 0.4M NaCl gradient. Gel tracks : [1] 30µg total yeast protein [2] total soluble protein [3] guanidinium-solubilised fraction [4] Zn Sepharose-purified [5] Q-Sepharose-purified [6] native pertactin [7] Amersham markers.

EXAMPLES

General information pertinent to the examples:

<u>Media</u>

YPD, 1 litre: 10g yeast extract, 20g peptone, 20g glucose.

YNBBG, 1 litre: 13.4g yeast nitrogen base w/o amino acids (Difco

LABs., Detroit, Michigan, USA), 0.4mg biotin, 20ml glycerol.

YNBBGCas: same as above plus 10g casamino acids.

YNBBD: same as YNBBG but 20g glucose instead of 20ml glycerol.

YNBBM: same as YNBBG but 5ml methanol instead of 20ml glycerol.

YNBBDCas and YNBBMCas had 10g casamino acids per litre.

Solid media: as above plus 20g of agar per litre.

<u>EXAMPLE 1</u> - Construction of <u>Pichia pastoris</u> intracellular expression vectors for P.69

The vector pPIC3-60.5K, deposited at THE NATIONAL COLLECTION OF INDUSTRIAL AND MARINE BACTERIA LTD., P.O. Box 31, 135, Abbey Road, Aberdeen AB98DG, Scotland, UK on 30th March, 1990 under Accession No. 40270 derived from pA0804 (Digan et al, Dev.Ind.Microbiol. 1988 29, 59-65) was used for intracellular expression of P.69 in Pichia pastoris. This vector uses the promoter from the AOX1 gene to drive expression and can be integrated into the host chromosomal AOX1 locus. facilitate insertion of the P.69 the gene synthetic oligonucleotides shown in Fig. 2A were cloned between the AsuII and EcoRI sites of pA0804, to give pPIC1. A derivative of this plasmid, pPIC2, which lacks the EcoRI site was then constructed. This was done by digesting with EcoRI followed by filling in of the protruding single stranded ends with the Klenow fragment of DNA polymerase I and ligating together the blunt ends. A 1.8kb EcoRI-NheI fragment (see Fig. 2B) from the plasmid pPERtac8 (Makoff, et al, Bio/Technology 8, 1030 (1990)) which contains most of the gene encoding P.69 (a 60.5kD polypeptide) was inserted into BamHI-SpeI cut pPIC2, using the adapter oligonucleotides shown in Fig.2A, to give pPIC3-60.5K. oligonucleotides encode the 5' end of the P.69 gene including the initiator ATG c don.

EXAMPLE 2 - Transformation of Pichia pastoris

<u>Pichia pastoris</u> strain GS115 (<u>his4</u>) was transformed with pPIC3-60.5K using the sphaeroplast method described by Cregg <u>et al.</u>, Bio/Technology 5: 479-485 (1987). Transformants were regenerated on minimal medium lacking histidine, so that His colonies would be selected. The transforming DNA was 10 or 20ug of BglII-digested pPIC3-60.5K. This digest contains two DNA fragments, one of which (7.2 kb) has <u>AOX1</u> sequences at either end, so that it is targeted to integrate at and replace ('transplace') the chromosomal <u>AOX1</u> gene.

The His transformants generated are mainly found to undisrupted AOX1, and transplacements (aox1) may be isolated using a further screening procedure. Transplacements may be identified by their slow growth on methanol (Mut as opposed to Mut phenotype; Cregg et al., Bio/Technology 5 479-485, 1987). The transformants can be picked directly off regeneration plates and tested for growth on minimal methanol plates (YNBBM agar). Alternatively, the regeneration top agars can be lifted and homogenised in water, and the yeast cells plated to about 300 colonies per plate on minimal glucose plates (YNBBD agar). Mut colonies are then identified by replica-plating In general, we and others have found onto minimal methanol plates. the proportion of Mut^S to be 10-20% of all the transformants. Occasionally Mut transformants might be scored as Mut and these may also prove to contain multiple copies of the vector.

EXAMPLE 3 - Screening for multi-copy transformants

In order to screen large numbers of transformants rapidly, they were grown in individual wells in a 96-place sterile microtitre plate. 200ul of YPD broth in each well was inoculated with a transformant, and the plates incubated for two days, at 30°C without shaking, to ensure growth to stationary phase. Included on each microtitre plate was a well c ntaining GS115 (negative c ntrol) and ne containing a known pPIC3-60.5K integrant (p sitiv contr 1). Using a multi-channel

pipett r, 50ul samples of each micro-culture were transf rred onto a nitrocellulose filter on a Schleicher & Schuell 'minifold' under vacuum. The filters were air dried, marked for orientation, then treated in the following way to lyse the cells: (i) 15min at room temperature with 50mM EDTA, 2.5% 2-mercaptoethanol pH9.0, (ii) 4hrs at 37°C with lmg/ml zymolyase (100T) in water, (iii) 5min at room temperature in 0.1M NaOH, 1.5M NaCl and (iv) twice for 5min at room temperature in 2 x SSC. Each treatment was performed by soaking 2 sheets of 3MM paper with the solution and placing the nitrocellulose filter on top. After these treatments the filters were baked at 80°C for 1hr.

The filters were probed by Southern hybridisation using P.69-specific DNA radiolabelled to high specific activity using random-primed labelling (Feinberg, A. and Vogelstein B., (1989), Anal. Biochem., 132, 6-13). Standard methods were used for prehybridisation, hybridisation, washing and autoradiography.

Fig. 3 shows the results of such a screen of over 200 Mut^S transformants of pPIC3-60.5K. All the transformants reacted with the probe and most gave a similar weak signal (single-copy transformants), however, a very small proportion gave much stronger signals. These multi-copy transformants were tested further. The positive control in the filter shown is a transformant previously identified as multi-copy.

The copy number of the P.69 structural gene was determined by quantitative dot blot analysis of the DNA (Table 1). SL3, SL4 (Mut⁺), SL18 and SL22 gave significantly higher levels of product than single-copy integrants, up to 5% of cell protein (Table 1). The majority (90%) of the pertactin produced by SL4 and SL22 was insoluble. SL 22 was deposited at NCIMB, Aberdeen, Scotland under Accession Number 40391 on 22nd March 1991, in accordance with the terms f the Budapest Treaty.

EXAMPLE 4 - Protein analysis

Selected transformants were cultured in YNBBGCas for 2 days at 30°C to reach stationary phase. These starter cultures were diluted to an 0D₆₀₀ of 0.25 in 5ml fresh YNBBGCas and grown for 6hr. To induce these cultures the cells were collected by centrifugation, washed once in sterile water, and resuspended in YNBBMCas. Inductions were carried out for 2 days at 30°C.

Cells were then harvested by low speed centrifugation, washed once in water and suspended in 0.5 ml ice-cold break buffer (20mM sodium phosphate pH7.0, 0.1% triton X-100, 4mM phenylmethyl sulphonylfluoride). Acid washed glass beads (0.45mm) were added and the cells were broken by vigorous vortexing. The protein concentration of the extracts was determined using the BioRad protein assay (BioRad, according to manufacturer's instructions) and the material was stored at -20°C.

Proteins were separated by electrophoresis in 7.5% SDS-polyacrylamide gels (Laemmli U.K., Nature 227: 680-785, 1970). The proteins were visualised in the gel by staining with Coomassie Brilliant Blue R. Alternatively the proteins were transferred to a nitrocellulose filter and reacted with the P.69 specific monoclonal antibody BB05 (Montaraz et al., 1985, Infect. Immunity 47, 744-751; deposited at PHLS Public Health Laboratory Service Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire, U.K. in the European collection of Animal cell cultures under Accession No. 90020103, on the 1st February, 1990 in accordance with the terms of the Budapest treaty), then with goat anti-mouse IgG conjugated to horse-radish peroxidase, and developed with H₂O₂ and 4-chloronaphthol (BioRad). In this way, the expressed P.69 could be specifically detected.

Fig.4 shows a Western blot of induced extracts of four selected multi-copy transf rmants (SL 3, 4, 18 and 22) and ne typical single-c py transformant. By comparis n to standard concentrations of

pure P.69, it can b estimated that single-c py transplacements express P.69 at 0.1-0.5% of total cell proteins (t.c.p.), SL22 at approx. 2%, and SL4 at approx. 5%. These levels are for shake flask induction conditions.

EXAMPLE 5 - Expression of P.69 during high cell density fermentation

In optimal fermenter inductions an improvement in protein yield may be observed. Thus SL22 has been induced in a controlled fermenter (Example 5) and expresses P.69 at about 10% t.c.p. SL4, which was subsequently determined to be Mut showed no change in expression levels in the fermenter. In low-expressing transformants the P.69 is largely soluble (approximately 55%), whereas at high expression levels it is mainly (approximately 90%) insoluble. This is preferable since the material can be readily isolated, renatured and purified from the insoluble fraction.

Production of P.69 by high cell density Pichia pastoris cultures was carried out using SL 22 in a 2L Braun fermenter equipped with monitors and controls for pH, dissolved 0_2 , stirring speed, temperature and air flow. A 10ml YNBBG overnight culture was used to inoculate the fermenter containing 1 litre of 5 x basal salts (phosphoric acid, 42mls/L; calcium sulphate 2H,0, 1.8g/L; potassium sulphate 28.6g/L; magnesium sulphate 7H₂O, 23.4g/L; potassium hydroxide, 6.5g/L) with 4ml of PTM_1 salts (cupric sulphate $5H_2O$, 6g/L; potassium iodide, 0.08g/L; manganese sulphate H₂O, 3g/L; sodium molybdate, 0.2g/L; ferrous sulphate 7H20, 65h/L; biotin, 0.2g/L; sulphuric acid, 5ml/L) and 5% (v/v) glycerol at 30°C. Dissolved oxygen was maintained above 20% by adjusting aeration and agitation, and the pH was maintained at pH5.0 by the addition of 50% (v/v) ammonium hydroxide. Growth was continued until the glycerol was exhausted (24-30hr). A limited glycerol feed (containing 50% w/v glycerol and 12ml/L PTM $_{\rm l}$ salts) was then initiated at 12ml/hr for 17-21 hr. After this period the culture was induced by replacing the glycerol feed with a methanol fe d (100% methanol plus 12ml/L PTM, salts) at 1ml/hr for 2hr. Then the methanol

feed rate was gradually increased over a period of 6 hr to 6ml/hr and the fermentation was continued using these conditions for a further 40hr. At this point the methanol feed rate was reduced to 2ml/hr.

Samples were taken from the fermenter at different times after induction, and were analysed for P.69 expression as described in Example 4. The results of a Western blot analysis are shown in Fig.5, indicating that levels of P.69 of about 10% of cell protein were achieved.

EXAMPLE 6 - Renaturation and purification of P.69

Induced SL4 cells were harvested, suspended in buffer A (50mM tris HCl pH8.0, 0.1M NaCl) + 1% triton X-100, and lysed with glass beads using a Bead Beater (Biospec Products, Bartesville, OK). Insoluble protein was pelletted by centrifugation, washed and suspended in 6M guanidinium thiocyanate/buffer A. On dialysis pertactin remained soluble while yeast proteins could be removed by centregufation. Solubilised pertactin was loaded onto a chelating Sepharose column charged with zinc and equilibrated with buffer A. After washing with 0.5M NaCl, pertactin was eluted in 50mM MES pH6.0, 0.1M NaCl. Pertactin was further purified by chromatography on Q-sepharose in 50mM tris HCl pH8.0, with elution using a 0 to 0.4M NaCl gradient. Overall recovery of pertactin was 40%.

EXAMPLE 7 - Protection Data

Recombinant <u>Pichia</u> derived P.69 was tested for its ability to stimulate protection by toxoid in the Kendrick test, which is the standard international potency assay for whole-cell whooping cough vaccines.

Vaccines, containing $5\mu g$ (TEST A) OR $20\mu g$ (TEST B) of toxoid, $20\mu g$ P.69 and 10% alhydr gel/PBS, were serially diluted in PBS and 0.5ml doses wer given intraperitoneally to male and f male NIH/S mice.

C ntrol mice r c ived whole-cell vaccine (British ref. 66/84; t p dose 8 IU/ml). After 14 days the mice were challenged intracerebrally with $20\mu l$ B.pertussis 18-323 (approximately $50LD_{50}$).

Table 2 shows the results of two experiments, comparing toxoid with or without added P.69. Toxoid alone was clearly less protective than the whole-cell reference vaccine and could not be improved by increasing the dose from 5μ g to 20μ g. Addition of native or yeast-derived pertactin increased protection to the level of the whole-cell vaccine. For comparison, P.69 produced from E.coli (A.J. Makoff et al Bio/Technology 8, 1030 (1990)) was also included in the test and gave similar results. The results with pure recombinant P.69 from two different sources show that the immunogenic effect was due to P.69 itself, rather than contaminating B.pertussis antigens.

Table 1. Expression of P.69 pertactin in r combinant Pichia.

| Yeast/plas | mid | Copy no.ª | Expression level b (% cell protein) | Solubility |
|------------|--------------------------|-----------|-------------------------------------|------------|
| pPIC3-P69 | (SL1,Mut ^s) | 1 | 0.5% | 50% |
| pPIC3-P69 | (SL18,Mut ^S) | 12 | <2% | nd |
| pPIC3-P69 | (SL3,Mut ^S) | 13 | <2% | nd |
| pPIC3-P69 | (SL22,Mut ^S) | 21 | 2% (10% ^C) | <10% |
| pPIC3-P69 | (SL4, Mut [†]) | 30 | 5% (6% ^C) | <10% |

^a The integrated vector copy numbers were determined accurately by dot blot analysis of purified DNA probed with radiolabelled P.69 structural DNA or DNA from the single-copy <u>P.pastoris ARG4</u> (supplied by K.Shreekrishna, Phillips Petroleum Co.). A transformant known to be single-copy from Southern analysis was used as a control. Hybridised probe was measured by scintillation counting.

b Shake-flask inductions.

C Peak levels in fermenter inductions.

Tabl 2.

| | Survivo | rs after c | hallenge ^a | |
|--|---------|----------------|-----------------------|------------|
| Antigen dilution | 1 | 1/4 | 1/16 | 1/64 |
| TEST A | | | | |
| Toxoid alone | . 10 | 2 | 0 | 1 |
| Toxoid and native P.69 | 14 | 8 | 0 | . 0 |
| Toxoid and E.coli recombinant P.69 | 17 | 8 | 1 | 0 |
| Reference vaccine | 15 | 7 | 1 | 1 |
| TEST B | | | | |
| Toxoid alone | 8 | 3 ^b | 1 | , 0 |
| Toxoid and native P.69 | 15 | 11 | 2 | 2 |
| Toxoid and <u>Pichia</u> recombinant P.69 | 15 | 10 | 3 | 0 |
| Reference vaccine | 15 | 7 | 2 | 1 |

a 18 animals per group

b 17 animals in this group

CLAIMS

- A <u>Pichia</u> microorganism transformed with DNA for the expression of a pertactin antigen whose amino acid sequence is at least 95% homologous with that set forth in Figures 1A, 1B or 1C, or an antigenic fragment thereof.
- 2. A <u>Pichia microorganism</u> as claimed in claim 1, wherein the amino acid sequence is at least 98% homologous with that set forth in Figures 1A, 1B or 1C, or an antigenic fragment thereof.
- 3. A <u>Pichia</u> microorganism as claimed in claim 2, wherein the amino acid sequence is substantially the same as that set forth in Figures 1A, 1B or 1C, or an antigenic fragment thereof.
- 4. A <u>Pichia</u> microorganism as claimed in any of claims 1 to 3, wherein the antigenic fragment essentially consists of the amino acid sequence:

PGPQPP; or APQPGPQPPQPPQPPAPAPQP

- A <u>Pichia</u> microorganism as claimed in any of the preceding claims, wherein the DNA is integrated into the <u>Pichia</u> chromosomal DNA.
- 6. A <u>Pichia</u> microorganism as claimed in claim 5, wherein multiple copies of the DNA are integrated into the <u>Pichia</u> chromosomal DNA.
- 7. A <u>Pichia</u> microorganism as claimed in claim 6, wherein between 5 and 30 copies of DNA are integrated into the <u>Pichia</u> chromosomal DNA.

- 8. A <u>Pichia</u> microorganism as claimed in any of the preceding claims, transformed with an expression cassette which includes the DNA encoding a pertactin antigen, or an antigenic fragment thereof.
- 9. A <u>Pichia</u> microorganism as claimed in any of the preceding claims, wherein the organism is <u>Pichia pastoris</u>.
- 10. A process for producing a pertactin antigen which comprises culturing a transformed <u>Pichia</u> microorganism as defined in any of the preceding claims.

| 6 6 L 6 A A P A A 6 6656666666666666666666666 | F L G T CITICIGGCACC 470 A80 | G A 'S I CCAGGCCAGCATC 590 600 | 0 8 L 0 GCAGTCATTGCAG 710 720 | T L D G TACGCTCGACGGC 830 840 | A G G A V P G G GCCGGCGGTGCGGTTCCCGGCGGT 940 950 950 |
|---|---|--|---|---|---|
| A L G A CGCTGGGCGC 220 2 250 1 5 G T T GCGGAACCACC 340 3 | I R R CATCCGGCGC | E G A CGAACAGGC BO | 75 | E L | A GG 6 A CCGGCGGTGCG |
| 7 | L S D D G 1 R R TGTCCBACGATGGCATCCGGCB 450 460 | Y V A G 1 A TG TGGCCGG 570. | G L H 1 1660:1160ATA1 690 | L G 16666 | G D A L 6 |
| CGCTGGCCATG 200 200 P G G V CGGGGGGTA | | G 1 A L | 1 V D G 17 CGTCGACGGG 680 | A A V S GGGGGTGTCT BOO | |
| R R I I L A M A 1 1 1 1 1 1 1 1 1 | V T S TGTCACGTCGT 430 | D D D SGACGACG | E R G A N V T V D R S A 1 V D G G L H 1 G A CGAGCUCGGCGCCAATGTCACGCACGCACCATCGTCGGGGGGCTTGCATATCGGCGCC 40 650 650 660 670 680 690 700 | , L R D T N V T A V P A S G A P A A V S V GETGEGEGEGEGEGEGEGEGEGEGEGEGEGEGEGEGEGE | I R A T I R R IGLGCGCGACGATACGGCGG 910 |
| | R N G S GGAACGGCAG 420 | G D 1 W SGCGACACCTGC | V T V 0 31CACGG1CCA 660 | V P A S 31GCCCGCLA(3) 780 | 1 V A A H Q G A V V H L Q iggtgccggccatgcatgcagggggggggggggggggggg |
| 1 V K A A A L L L L L L L L L L L L L L L L | L 0 1 CTGCAGT | > ⊢ | G A N CGGCGCCAATG | CB16ACCGCCC | 6 A V AGGGGCGGTCC 890 |
| M S L S R CATGTCTCTGTCACGG O 160 K 1 G E R CAAGACCGGTGAGCGG | P A A E CCGCGAG | D H A T L A N CGATCACGCCACGCTGGCCAACG 520 530 | → ← | R D T N Gegacacear 760 | A A M O SCGGCCATGCA 880 |
| H N H ISO ISO I V K I V K ATCUTCAGGA | L E · N P · SICTAGAAAATCCCG | | 6 6 V 0 166CGGCGTGCAGA 630 | v v L 367667667667 | A G V AGCGGGGGTGG 870 |
| CTCTCCACCTGGCATCCA 130 140 7 W N D S D W N N D S GACTGGAACCAGTCC | D A D G 1 L 2AGGCCCAGGGCATCCTG 370 380 | A B K L CCGCCAAGCTG SOO | L Q G A 19CAGGGGCT 620 | P 5 8 CGCCCAGCGG 740 | 6 6 R A 6CGGGCGGC/ 860 |
| CGTCTGCTCCCACTGGCATCCAATGAA 130 140 15 15 16 17 17 18 18 19 19 19 19 19 19 19 19 19 19 19 19 19 | G R Q A Q G 1 L L E GGCCGTCAGGCCAGGGCATCCTGCTAGA 370 380 39 | V T V K A B K L V A GTCACCGTCAAGCTGGTCGCTAAGGCCGGCAAGCTGGTTGGT | A D S T L Q G A G G GCCGACAGCACCTGCAGGGCGTGGCGG 610 620 620 63 | P E D L P P S R V V CCGGAAGACCTTCGCCCAGCCGGGTGG1 | G H I T G G R A A G GGGCACATCACCGGCGGGCGGCAGGGG B50 B60 E |

Fig. 1A (cont i)

| 1 9 1 E 1 G G A R R F MGCCATCGAGACCGCGCGCGCGTTT 10 1180 1190 1200 | PVKLTLT66AD CCCGIGAGCTGACCGGGGCCGAT 290 1300 1310 1320 | N W T G A T R A V D B SATGGACGCGTACCCGCGGTCGACTCG 10 1420 1430 1440 | FOQPAEAGCTGGGCGGTTCAAGGTC | I D A S G O H R L W V GGACGCCAGCAGCAGGCTGTGGGTC 10 1660 1670 1680 | V K D G K V D 1 G T V NCANGGACGGCAAGGTCGATACCGTACCTAT | P Q P Q P Q P Q P Q P Q P Q P Q P Q P | A S T L W Y A E S N A IGCCAGCAGCAGCGC 2010 2030 2040 |
|--|--|--|--|---|---|---|--|
| 9 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 | 37.00 129. | A 2000 1410 | 70.5 | Н 0 D 1 ТССАССА 1650 | - A A A L L L L L L L L L L L L L L L L | | 166CC 2010 |
| E A P E L G A A 1 R V G R G A R V T V P G G S L S A P H G N V 1 E 1 G G A R R F E GAGGECECECECAGAGACCGGCGCGCGCGCGCGCGCGCGC | A P G A A P L S I T L G A G A H A G G K A L L Y R V L P F V K L I L I G G A D GCGCTCAAAGCGCCCGCGCGCCCGGCGAACCCGGGGGGGG | A G G D 1 V A T E L P S 1 P G T S 1 G P L D V A L A S O A R W T G A T R A V D S GCOCAGOGOGOGOGOGOGOGOGOGOGOGOGOGOGOGOGOG | L S 1 D N A T W V H T D N S N V G A L R L A S D G S V D CTGTCCATCGACACGCCACCTGGGTCATGACGGACAACTCGAACGTUGGTGCGUTACGGUTGGCTAGCGACGGUAGCGTCGATT 1430 1460 1470 1480 1490 1500 1510 1520 | L T V N T L A G S G L F R M N V F A D L G L S D K L V V M D D A S G D H R L CTGACGGTCAATACGCTGGGGGGGGGGGGTGTTCCGCGAACGTGGGGCGGGC | R N S G S E P A S A N T L L V U T P L G S A A T F I L A N K D G K V D I G T V CGCAACAGGGGGAGGCGGCCAACACGCGGCGACACGTGGTGGTGGTGGTACCTAI | RYRLAANGNGOOT GOWSLVGAKAPPAPKPAPGGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGC | P E A P A P A G R E L S A A A N A A V N T G G V G CEGGAAGCGCCGCCGCGCGCGCGCGCGCGCGCGCGCGCGC |
| 6 6 6 6 | 9000 | 6000 | L CTGT | CTGA | R CGCA | COCT | 6000 |

Fig. 1A (cont. ii,

| 00 | ມ ຸ | മറ | ဗုဝ | 90 | . 60 | υo |
|---|---|---|---|---|---|--|
| , , 46616 2160 | → H CCA 228 | 240 240 | R CCG 252 | 1 R 264 |) CCT 276 | 1 286 286 |
| S A A | 992 | 200 | ATT | 6 6 9 | 1666 | 3616 |
| 900 | 999 | E SC S | 7007 | 600 | | 200 |
| 100 E | 2270 | 9 6 | 1660 2510 | - rear 2630 | 27.50 E | 4 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 |
| SCT. | | , 200 | 1 36C1 | a de | 3000 | 361 |
| 3000 | 7 | , > | 900 | 100 | 1000 | . × |
| 140 | 7 260 | 7 E 8 | 3000 | 8 6 6 7 0 6 2 0 | 6 6 740 | 6 60 T 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 |
| A 200 | 6 6CT 2 | D ACT | 2 2 2 | A GC | 7 16CG 2 | A 2006 2 |
| | <u>6</u> .00 | 2 S T G | ## ## ## ## ## ## ## ## ## ## ## ## ## | : : :::::::::::::::::::::::::::::::::: | | ₹-% 906 |
| N Q S | 3ACC 250 | E 3AGA 370 | 150 140 | 34CG | ВААС 730 | 1. F. B30 |
| 200 | <u> </u> | 2 <u>2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 </u> | ַבָּיאַ הַאַנוּ | 5 AG(| | =- Å |
| - <u>1</u> | ¥ CCC | ۳ (9) | 3 E | ر د تق | 8 E | 3 E |
| O CAG | - A C 6 | 29 G | 99 | ၁ ဥ ဌ | ± 20 5. | - 133 6 |
| 0 12 | .⊤AT: 22 | A 33 5 23 23 23 23 23 23 23 23 23 23 23 23 23 | 2 P 2 | 28 | 4 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 | 7 A 16 |
| F 75 | 999 | 2002 | 4 کی | 8 185 | 191 | ٠ ا |
| 10 a | 300 € | ານຄຸ | 1 U O | 900 | 10.00 10. | 36.1 |
| CGC 21 | 100 22 | 1 6AC 23 | 7 T PC 24 | | N 11.0A | 7 4 2 8 2 8 2 8 2 8 2 8 2 8 2 8 2 8 2 8 2 |
| | 999 | _ D D | 7.10 | > 199 | ב אם | , 133 133 133 133 133 133 133 133 133 133 |
| | 9990 | 959 | ¥ 55 c s | ຶ່ນຕີ | , TAC | , e 9999 8099 |
| 3 210 210 | 222 | 23,52 | 24¢ | 2 2 2 3 3 5 5 5 6 7 6 7 6 7 7 7 7 7 7 7 7 7 7 7 7 | 276 | 200 |
| 3666 | 300 | [2 | 4 J | 9 9 9 | 0 T | > PCT |
| CCT | 8 0 11 0 | - <u></u> | F. 60 | Б. А. А. С. | A 50 50 | е ВАG Т |
| 6 209 209 | 6 1221 | 5 233 | 1. 249 | 3ACG 23.7 | 6 36C6 265 | 7 7.00 2.81 |
| 9900 | ອ | D SACK | ອ | . & 33 | <u>م</u> کی و | 1031 |
| م کن م | ٥٥٥ | م کرن | و تا | > p 0 | 7 E 0 | 0 0000 |
| 208(C | 220 220 | 1 ATC 232 | 6 66C 244 | я С66 256 | £ бас 268 | , 11A1 280 |
| ٦ ا | ₹ છે | > I | > 0 010 | ב פר | ၁မွ | , C 16 |
| Z P Z | >610 | - PC C | 9907 | 98 | 755 | s di C di |
| 7 20.02 | A 200. | 76CC 231C | 74 X | 2 4 8 8 8 8 8 8 8 | 2676 | 200 H |
| 8 DE . | ΞĞ., | ≻ (| - CAC | € 00 | 2 D | 9 99 |
| DI I | ۵ (| 9 9 9 9 | . & 9 | € D | 6 D | E 00 |
| 769 060 | 4 D 0 | 566 300 | 420 | 2 D & | X 6 0 | .666 .780 |
| 9 9 7 | 999 | 761 | 7 A 4 W | 7 6 7 7 | CA1 | 75 |
| ביר ביר | L | , gC , | 999 | 3166 | AT W | , 55 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 |
| 4 350 350 | 756 170 | 36.0 29.0 | 10 P | 8 830 | 0 90 90 90 | A |
| 2 | 9 - 1 22 | A C P | A C C C C C C C C C C C C C C C C C C C | _ 00 000 7 | 760.2 | H 2 |
| L S K R L G E L R L N P D A G G A W G R G F A D R O D N R A G G R F D G K V TTGTCCAAGCGCCTGGGCGTGGCCTGGGCGGGGGGGGGG | A G F E L G A D H A V A V A G G R W H L G G L A G Y T R G D R G F T G D G G G H G CCGGCTTCAGCTGGGCGCCGCGGCGGCGGCGGCGGCGGCGGCGGCGGCG | T D S V H V G G Y A T V I A D S G F Y L D A T L R A S R L E N D F K V A G S D G Accoacagotocatotogogoctatoccacatatogocoacagogottulacutogoacocagococcagocutofoagaaaatoacttcaagotogocogoacogogo 2290 2300 2310 2320 2330 2330 2340 2350 2350 2300 2300 2380 2390 2400 | Y A V K G K Y R T H G V G A S L E A G R R F T H A D G W F L E P D A E L A V F R Tacgcggtcaagggcaaggacccatggggggggggggggg | A G G G A Y R A A N G L R V R D E G G S S V L G R L G L E V G K R 1 E L A G G R GCCOGCGGCGGTGCGTACCGCGCAACGGCCTGCGGGGCGCGGCGGCGGGGCGCGGCCTGGGCCTGGAGGTGGCGGAAGCGGATCGAAGTGGCGGCGGCGGAGCGGCGGCG 2530 2540 2650 2550 25./0 2580 2590 2600 2610 2620 2630 2640 | O V O P V I K A S V L O E F D G A G I V H I N G I A H R I E L R G I R A E L G L CAGGIGCAGCCAGCAGCGCGCGCGCGCGCGCGCGCGCGCG | G M A A A L G R G H S L Y A S Y E Y S K G P F L A M P W T F M A G Y R Y S W ¢ OGCATGGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGC |
| - - | ف | ∢ | - | ט | Ü | U |
| | | | | | | • |

Fig. 1B.

| 5 6 | m 0 | u o | សត | | 12.0 | u 6 | ⊢ ∩ |
|---|---|--|---|--|---|--|--|
| 2 2 | ح يَنِ جَ | พลิลั | + 7966 480 | - E 04 | 9 6 6 | ວ <u>ດີ 8</u> | 1088 |
| 99 | ون ۵ | > 5 | ့ မမ္မ | a p | 75 | οğ | ၂၀ ဗ္ဗ |
| ē | r D | . × ₫ | غ د ق | φù | 6 5 | ۶Ē | 46 17 |
| ນ | a Li | - u | 7 2 | 990 | 2 to 0 | - L L | To E a |
| 5 = | 200 | d in | . i. 6 | Ü | 71. | 4 P | 5 5 |
| 5 | * <u>B</u> | P D | 6 D | 4 D | . בנר | יבר ר | , 5 |
| נפ | ဗၓ္ဌ | .► Ŭ | ¢ 2 | æ ₹ | ⊢ Ü | m <u>é</u> | 0 8 |
| פֿבפ | 25.6 | ခဋ္ဌိဇ | > 50 | a ge | <u>ه ازا</u> م | z ĕ C | ြစဗ္တို |
| 8 0 | ÄÜÞ | - Üğ | ດ ຄື 4. ກິ § | | - 1 5 | 4 Ü 🛱 | 4 2 8 |
| 1 | | ຊິນ | 9 E | 4 D | 1 d | ם ם | 7 7 |
| 1C11CC1811CCA1CGCGG1GCGGCCA1GGCGGG | . 8 | _ 2 | Ö | 25 | 5 | | 00 |
| يق ق | م تا ۔ | _8_0 | وثا | و آ | -0.0 | e_o. | |
| - P | . g 2 | e p v | 7 7 7 8 4 5 | 7 T 8 7 8 9 7 8 9 7 8 9 9 9 9 9 9 9 9 9 9 9 | 0 000 | , <u>1</u> 2 | 250 |
| · Ē — | 1 4 | > 10 | ن د | ֝֝֝֝֝֝֝֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֡֓֡֓֓֓֓֓֓֓ | ဗ ဋ | พัก | ם פ |
| 16C1GCGCCCA1 | SLSRIVLAAPLRRITLAAMALGALGCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC | 9 25 | S G Q L F D E G V R R F L G I TCGGGACAGCTGTTCGAGGCGTCCGGCGTTTCTGGGCACC 440 450 460 480 470 480 | € 55 | 9 5 | > 5 | ۳ <u>ي</u> |
|) 6 | 758 | ٠ <u>ن</u> و | ၁ ဗ္ဗီ ဝ | - 50 | > = 0 | ∢ ပို့ ဝ | Ξ Β΄ Ω |
| 5 | ~ § % | _១ ដូក | 8 7 4 | مَنْ وَ و | | 4 2 8 | - 4 6 |
| ភ្ជ | ► U | 9 4 | 2 T 1 | 9 C G | F U | 200 | F 13 |
| _ | Ä | Ü | _ 5 | CG | 200 | ב פרי | 20 |
| 50 | ្ព ភ្ជុំ | , 6 o | _ A & | ္မွင္တိုင္ခဲ့ | 2 6 6 | a no c | ្តភ្លូ |
| 5 | E 55 - | B မွ n | > 5 5 | កម្ពុ | ភពិ | 9 22 7 | E D |
| Ī | ה ה | × g | ທ ອີ | ie ij | ₽ ₹ | ນ ຕິ | ت ق |
| Ę | • !! | 4 0 | ဗ ပ္ပ | الم ا | > ដ | ∢ ដូ | 7 5 |
| 5 3 | 4 11 8 | 1 4 0 | Z O O | 0 4 4 0 6 0 | + n 6 | • ក្តី និ | 140 |
| 100 | 4 D _ | בַבְּי | 9 9 | 8 D D | ي ئ | , בני פני | > 5 |
| ន្ទ | 99 | a ii | ຼິ່ນ | Š | 25 | ິນ | .0 |
| ב | 15 | | . 5 | 25 | ^ 8 | 39 | 7 6 |
| 500 | > 107 | 1 4 0 2 0 0 | R D 0 | ZEO | A 75 8 | 3AC 770 | 4 2 5 6 8 9 6 6 |
| ũ | - 4 | a <u>4</u> | ֓֞֝֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓֓ | ٠ <u>ن</u> | ٠ ق ٢ | > 5 | ဗဗ္ဗ |
| 6 | E 2 | # 5 | e GAG | 75 | ۳ <u>ي</u> | ນ ນີ້ | a g |
| Ď | งก็ ช | E SAG | ۸۵۷ | ۲ کا | a ga | ⊢ Ŭ | I 5 |
| 40 40 | 75 4 | ۾ واو | 4 7 6 | 200 | > t 6 | 7 P C P | 4 2 8 |
| 513 | 8 7 | 9 | | Ž | 65 | 200 | 60 |
| ŭ | - 5 | ٥ | 7 5 | _ 5 | ້ ຮູ | 0 00 | ຸ ອິ |
| 30 | - E o | 180 | * d o | 200 | 750 | ס מיר | 250 |
| d b | H N NTGARC | 18.7 27. | A S & | 916 | ខេត្ត | 7.5 | 9 8 6 |
| 99 | £ 4 | - F | 75 | > 5 | ల ల్ల | > 5 | ون ۲ |
| ě | Š | ຮຸບ | 15 | 75 | ۵ ي | æ 👸 | م يُ |
| 17 AA(| 40 | B 4 0 | > 0 | x & o | ە تا ھ | ه و تا ه | 4 B 0 |
| 767 | 56CA1 | ŽŮŔ | ្តប្តូក | ္ကပ္သိန္တ | 65 | 27.4 4.7 | ္ကပ္က စီ |
| ដ | ָ בָּ | ر ک | _ <u>5</u> | ָם - נו | 20 | _ 55 | _ <u>_</u> |
| G1 C | באכ | * 6 | , i | 900 | ניי | 100 | 992 |
| ט ב | 1777 | 3 5 0 | 4 <u>5</u> 0 | ¥ ₫ o | ⊢ Ü o | 7,50 | ې ق |
| 1 dg | 57 | 70.00 | e gig | 750 | 2 D S S S S S S S S S S S S S S S S S S | 967 | A 10 |
| Į, | <u>5</u> | - Ü | α <u>5</u> | ► Ü | o Š | 7 E | ΣĞ |
| NTCGATGATGCGTCGCTGTAACACGGCAAATACCGTGCATTGCAGCGGTTCTGGGTGGTGTTCTTCGTACGT 1 20 30 40 50 50 70 | H N H COTCTCTTCACCCGGCATCCAATGAGCATG | V A D W N N Q S I I K A G E R Q H G I H I K Q S D G A G V R I A I G I I K V S Iacgcegatiggaacaaccagiccaicaacggceggceggcagcacacggcaccaccagcaaccaac | G R G A G G V L L E N P A A E L R F G N G S V T S ;GTCGTCAGGCCGTCCTGCTGGAAATCCCGGGCCGAGCTGCGGTTCCAGAACGGCAGCGTCAGGTCT 370 380 380 430 400 400 | V T V K A G K L V A D M A T L A N V S D T R D D G 1 A L V V A G E Q A Q A S 1 Heacegteaaggeggaaggeggegggaeggegggegaegaegaegae | A D S T L G G A G G V R V E R G A M V T V Q R S T 1 V D G G L H I G T L Q P L G ICCGACACCACCCTGCGGCGCGCGGGGGGGCGCGGGGCGCGGGGGGGG | PEDLPPSRVVLGDTSV1AVPASGAPAAVSVFGAVFGANELTVDG COGAAGACCTTCCGCCCAGCGGGGGGGCGCCCGCGGGGGGGG | G H I I G G R A A G V A A H D G A I V H L Q R A I I R R G D A P A G G A V P G G IGCACATORACEGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG |
| - | ñ | <u>-</u> | • | | - 74 | - n | m |

4/13

SUBSTITUTE SHEET

Fig. 1B (cont. i)

5/13

SUBSTITUTE SHEET

Fig. 1B (cont. ii.)

OVAVKGKVRT HOVGAS LEAGRRFA POOT SELEAGRE E AN ADÓWFLE POAFLE POYF GGGTACGCGGTCAAGGGCAAGTACCGCACCATGGGGTAGGCGCCTCGAGGCGGGCCGGCTTCGCCCATGCCGACGCTGGTTCCTCGAGGCGGAGGCGAGGCGAGGCGGTGTTC 2410 2420 2420 2430 2440 2450 2460 2470 2480 2490 2500 2510 2520

6/13

H V G G G S Y R A A N G L R V R D E G G S S V L G R L G L E V G K R I E L A G G CGGTCGGCGGCGGTTCGTACCGCGGCCAATGGCCTGCGGTGCGGACGAAGGCGGCTGGTGCTGGGCCTGGGCCTGGAGGGTCGGCAACGGCAATGGCAACGGCGGC 2930 2940 2550 2560 2550 2550 2550 2580 2590 2600 2610 2610

Fig. 1C

| 10 20 30 40 50 60 70 80' 90 100 110 120 | 20 | 20 | 4 | | o S | 200 | 9 | 20 | ב - - | 90 | 200 | 100 | ยายายา | 110 | 120 |
|---|-------------------------------------|--------------------------------------|--------------------------|-----------------------|--|-------------------------------------|----------------------------------|--|--|---------------------------------------|---|-------------------------------------|----------------------|--|----------------------------|
| H N H S L TGCTCTTCACCCGGCATCCAATGAACATGTCTCT 130 140 150 | I 140 | H N H S ATGAACATGTCT 150 | . s . itctctgt 160 | S R CACGCA | 1 V 111GTC# 170 | V K A A P 1GTCAAGGCGGCGCC 170 | A P L 3CGCCCC1 180 | L R R TGCGCCGC 190 | TT | L A ACTGGCC 200 | Н А L ATGGCGC1 210 | . G A L GGGCGCCT 220 | ר G A זהפהרהרו | A CGCGC 230 | P A A ccccccc |
| A D W N N Q S 1 1 K A G CCGACTGGAACAACCAGTCCATCATCAAGGCCGG | N N G S 1 NACAACCAGTCCATE 260 | 1 1 K A 1CATCAAGGCC 270 | A G A G 280 | E R JAGCGCC | G H G CAGCACGGCA 290 | - 5 | H 1 L CACATCAC | к 0 S АССАААСС 310 | D G CGATGG | A G CGCCGGC 320 | H 1 K Q S D G A G V R T A T G T T I CACATCAAGCAAAGCGATGGCGCGTACGGACGGCCGCAT 300 310 320 330 340 350 | A T (GECACEGI | G 1 1 GAACGAC | I K Catcaagg 350 | K V S CAAGGTAAGC 360 |
| R Q A G G V L L E N P A GTCAGGCCGTCCTGCTGGAAAATCCCGC 370 380 390 | 6 V L L 38001CC1GC1G(| ENPA IGGAAAATCCCGC 390 | P A 1000 | A E GGCCGAGC OO | L R 2166661 410 | F Q FTCCAGA | N G S AACGGCAC 420 | F Q N G S V T S S G Q ICCAGAACGGCAGCGTCACGTCTTCGGGACAG 420 430 440 | S S STCTTC | G G G G G G G G G G G G G G G G G G G | L 2161 | F D E G V | V R R V TCCGGCGCT | . 11.0 | . G 1 GGGCACC . 480 |
| T V K A G K L V A D H A CCGTCAAGGCCGGCAAGCTGGTCGCGATCACGC | G K L V GCAAGCTGGT 500 | CGCCGAT | | T L IÇGCTGG | Т L A N V САСССТ GGC СААССТ 20 530 | v s stcaece s | / S D T F TCAGGGACACCC 340 | R D D G I A GGGACGACGCATCGCG SSO S60 | D G | ו א האוכה 560 | CTCTATGI | A G G GCCGCG(G) | E Q A | / A G E Q A G A S I GGCCGGCGAGGCCCAGCCAGCATC 380 590 600 | S 1 IGCA1C 600 |
| D S T L Q G A G G V R V Acagcaccctgcaggcgcgggt 610 620 630 6 | а 6 A 6 САGGCGCGGG 620 | 6 6 V R V 3GCGGCGTGCGGGT 630 6 | R V 3CGGGTCG 640 | E R JAGCGCG | 6 A N 3GCGCCAAT 650 | > 5 | 7 V (NCGGTCC# | 4 R S AACGCAGC 670 | TACCAT | 0 V CGT1GAC 680 | T V Q R S T 1 V D G G L CACGGTCCAACGCAGCACCATCGTTGAGGGGGGTTGAGGGGGGTTGAGGGGGGGTTGAGGGGGG | H 1 (CATATEG | G T L | - Н 1 G T L G P 1GCATATCGCACCCTGCAGCCGC 700 710 | L G 16CAG 720 |
| E D L P P S R V V L G D AAGACCTTCCGCCCAGCCGGGTGGTGCTGGGCGA 730 740 750 7 | PSRV CCAGCGGGT 740 | R V V L 266616616C16 750 | G D 166CGACA 760 | T S CCAGCG | V 1 31GACCG 770 | A V SCCG16C | ר א פ בכבפבכאנ 180 | T S V I A V P A S G A P A A V F CACCAGCGCGCGCGCGGCGGTGTT | A 4))))))) | 66CGGTG | V F 1GTATT 810 | G A N E L CGGGGCCAATGAGCT 820 | N E L ATGAGCT | T V TACGGTTG 830 | D G TGATGGC 840 |
| 4 I T G G R A A G V A A ACATCACCGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG | G R A A GGCCGGCCAGC | 4 G V CGGGGGTG 870 | | M D ITGGACG | M D G A 1. Catggacgacgat Bo B90 | > CG T G | | u в А Абсбсбс 910 | T 1 3ACGAT | R R ACGGCGC | GCAGGGGGACGACGGGGGGGGGGGGGGGGGGGGGGGGG | CCTGCCG(| 6 6 9 6066160 | V P GGTTCCAG | 6 6 655661 |
| V P G G A V P G G F G P Trecesces of tree G C F G F G F G F G F G F G F G F G F G | A V P G CCGTTCCGG(| 3 G F 3CGGCTTC 990 | 0001 | :TCC7.16 | D G W Y SACGCTGGTAT 1010 | . W Y 10601410 | 6 v 1 36C6166/ 320 | G | W C - | T V CACCGTC | S T V D L A Q S 1 V E A P Q L CACCGIGGACCICGCICAGICGAGGCGCGCAGGT 1040 1050 1060 1070 108 | O S ICAGTCGA 1060 | 1 V E TCGTCGA | E A P 466666661 | G L AGCTG 1080 |
| | | | | | | | | | | | | | | | |

7/13

Fig. 1C (cont. i)

| | ~ | | 8/13 | | | |
|---|---|--|--|---|--|---|
| 4 Ω 8 | 2 A C | 0 P O O | 0 4 0 6 0 1 | ວ ວິ | ه ځ د | 1 4 8 8 |
| 12.0 | | 100 | > 50 | 5 5 16 | 7 3A⊺ 18 |) 61 100 |
| ַלַ בַּ | 999 | R LI | ¥ ± | Z 0 | 70 | |
| | 29 | . 25 | _ 68 C | _ Š | 25 | |
| 7,00 | 999 N | 700 | 125 | 2334 | 7.00 | 910 |
| 111 | a 6 4 | S CTC | , 199 | , 100 1 | 7 Y T | |
| αĎ | ₹ ਹੁ | 0 Y 9 | 7 & G | 3 C | TAC | a 23 |
| ۳ <u>ن</u> ي و | 9 00 0 | 201 < | 31.10 | 3CT 04 | စ္ပင္သ | I F Ü Ö |
| Φ Ω = | 0 0 0 0 0 0 0 0 0 | 4 D 4 | 8 D V | 7 A C 1 6 C | 171 | |
| ត ទី | ٩٥ | * <u>)</u> | ລ ອີ | ıβ | 0 (g) | 6 A |
| ອິດ | , C16 | ۲- <u>۲</u> | 4 D | a Si | > 19 . | 1 " !! |
| - 10 C | ACG 290 | 4-10-14 0-14 | - M- 6- 15 | 0.0.0 | X 6 7 | .0-0-6 9-6-6 |
| + 50 1 1 | د د ت | 0 0 1 | A D D 1 | 5 AGC 1 | 6 767 | ↑ • 8 = 1 |
| a BAG | A A | - D | - 1 U | A D | O O | 0 A |
| A 1 C(| > E B 0 | 3 T S | 20 20 | л 5АС 40 | A A G | |
| >15. | 70.2 | 7 H H H H H H H H H H H H H H H H H H H | G 63 20 € | 7 166 | A P C | 6 18 |
| N Q | e SAG(| و ي | ٦. | 1 5 | A D | ♠ • Ö |
| ့ ပို့ | 4 D | 9 | ο 4 | , ŭ | ٦ <u>+</u> | 9 8 |
| T S S | 1. 1.270 | S 2000 | > 37CC 151CC | > 10 163 163 | 1 7 1 1 1 1 1 1 1 1 1 | 1 6 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 |
| ₽ 55 | > 15 | ارن و | ສິ້ | ٠ <u>٠</u> ٠ | 411 | ون ۲ |
| ΑÜ | F 50 | . T GC | _စ ည္တဲ့ | × 4 | ⊢ 00 | 7 11 |
| ឧ ភូពិ ស | > 4 4 | ₹ 200 | a 4 0 | 20.00 | 4 13 6 | × 60 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 |
| 16T | 161 12 | > 5 | 5 600 13 | 8 600 16 | A C C C C C C C C C C C C C C C C C C C | 70. |
| SCT | , 160 | D ACG | A CCA | TGA | 8 6CG | A CGC |
| 6 6 6 6 | A CGC 0 | ر و ر زو | ر 166 0 | 0 0 0 | o GCA O | ٦ 550 |
| 6 6 1 1 3 | R GGG 125 | 666 137 | 8 660 149 | L 166 161 | R GAG 173 | ا دور 185 |
| ິ ຫຼື ຫຼື | 6 66A | 200 | ار 160 | ACC | r CAC | A CCC |
| . 19 | 99. | ິ່ນ | _ ນ ອ | 600 | 1000 | A A G G |
| .98. 130. | \$ 0 C | 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 | . 50 0 80 0 80 0 | . n o | 20 A | 4 1 0 6 4 6 0 |
| _ 96 ± |) 99 11 | 15. | ۲ | | 757 | ישנפי (|
| 7 199 | 1 55 | 200 | _ [9] | 19 1 | 99 | . , , , , , |
| | 200 | 980 | | 190 | 150 | . 55 ° |
| 111 | 123 | , 5 t | 15.0 | 1 6 5 1 1 8 2 1 | 7 171 171 | , 100 100 100 100 100 100 100 100 100 100 |
| . 9 9 | 9 00 | בא | . 6 6 | e S | r F J | GAG |
| R 000 | G CA G | 755 | 0 68A | GTT | ָר אַנ האָנ | _ 6 4 6 |
| B | L 220 | 340 | 1 GAC 460 | 66.1 580 | CAA 700 | GCA 620 |
| 96€ | ⊢ ਹੁੱਜ | SCT. | CAT | 999 | 0 0 1 | 166 |
| ۳ Ö | 1 PE | 5GA(| 3G T. | ស ឬ ជ | ស <u>ថ្</u> ង | Z G |
| 1 6 1 0 1 0 | STC(| JAC(30 | Z E | 999 | Α Ω 0 | 9 9 9 0 1 |
| A A I R A G R G A R V T V S G G S L S A P H G N V I E T G G G A R R F P P A C CGCGCGCGCGCGCGCGCGCCCCCCCCCCCCC | PLSITLGAGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG | V A T E L P P I P G A S S G P L D V A L A S Q A R W T G A T R A V D S L S I D TCGCGACGACCCACCACCAGCCAGCCAGCCAGCCAGCCAG | A T W V M T D N S N V G A L R L A S D G S V D F Q Q P A E A G R F K V L M V D CCACCTGGGTCAACGTCGAACTCGAACTCGAACTCGAACTCGAACTCGAACTCGAACTCGAACTCGAACTCGAACTCGAACTCGAACTCGAACTCGAACTCGAACTCGAACTTGAAGGTCCTGATGGTCGAT 1450 1550 1550 1550 1550 1550 1550 1550 | L A G S G L F R M N V F A D L G L S D K L V V M R D A S G Q M R L W V R N S G TGGCGGGTTCGGGGCTGATGATGTCTTCGCGGACCTGGGCTGAGCTGGTCGTCATGCGGGACGCCAGCGACACAGGCTGTGGGTCCGCAACAGCGGC 1570 1580 1540 1600 1610 1620 1630 1640 1650 1650 1650 1650 1680 | E P A S G N T M L L V G T P R G S A A T F T L A N K D G K V D 1 G T Y R Y R L AGCCGGCCAGCGCAACACCCATGCTGCTGCAGGCCACGAGGCGCGCGGCGACCTTACCCTTGCCAACAGGCGGAAGGTCGATATCGGTACCTACC | A N G N G Q W S L V G A K A P P A P R A P G P G P G P G P G P G P G P G P G P |
| <u>د تا</u> | 7 2 | > 5 | αü | 7 L | n O | ∢ Ω |
| | | | | | | |

8/13

SUBSTITUTE SHEET

9/13

Fig.1C (cont. ii.)

R G F T G D G G G H T D S V H V G G Y A T V I A N S G F Y L D A T L R A S R CCGCGGCTTTACCGGCGACGGCGGCGACACGGCGTGCATGTCGCGACACACCGGTTTGTACCTGGACGCGACGCGTGCGGCCAGCCGC 7790 710 710 2320 2330 2340 2350 2350 2370 2380 2400

N D F K V A G S D G Y A V K G K Y R T H G V G V S L E A G R R F A H A D G W AAATGACTTCAAGGCGGCGGCGGCGGTTCGCCCATGCCGACGGCTGG 2410 2420 2430 2440 2450 2450 2510

G K R I E L A G G R Q V Q P Y I K A S V L Q E F D G A G T V R T N G I A H R CGGCAAGCGCATCGAACTGGCAGGCGGCAGGCGAGCCATACATCAAGGCCAGCGTTTGCAGGAGTTCGACGGCGGGGTACGGACCAACGGCATCGCGATCGC 2650 2660 2670 2680 2690 2700 2710 2720 2730 2740 2750 2760

L R G T R A E L G L G M A A 1, G R G H S L Y A S Y E Y S K G P K L A M P W ACTGCGCGGCACCCCCAACCCGCGCCATGCCGTGCTATGCCTCGAGTACTCCAAGGCCCGAAGCTGGCCATGCCGTGG 2770 2780 2790 2800 2810 2820 2830 2830

10/13

Fig.2A.

Asull BamHi

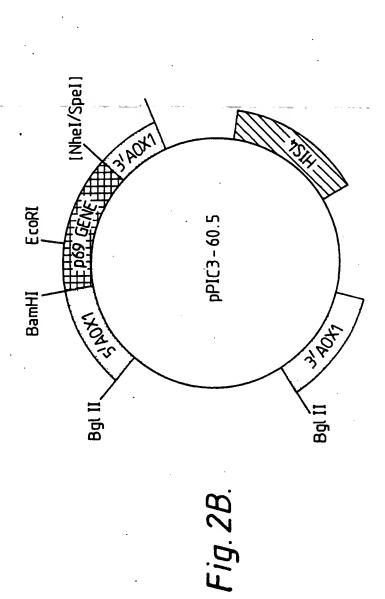
Spel

> -TAAGCGGCCGCG -ATTCGCCGGCGCTTAA EcoR I AsuII **EcoRI** BamHI SpeI EcoRI AsuII pA8884 pPIC1 Asull from pPERtac8 **EcoRI** NheI p69 GENE BamHI SpeI **EcoRI** (NheI/SpeI) EcoRI BamHI pPIC3-60.5K pPIC2 AsuJI

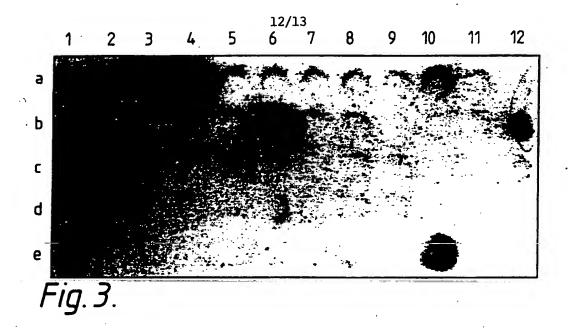
GATCCAAACGATGGACTGGAACAACCAATCCATCGTCAAGACCGGTGAAAGACAACACGG

GTTTGCTACCTGACCTTGTTGGTTAGGTAGCAGTTCTGGCCACTTTCTGAAGTGCCTTAA

Bamh I



-GGTGGTGTAGGCCTTGCAAGTACTCTGTGGTACGCTGAATCTAATGCATTATCTTAAGGATCC GCTAGC Nhei ECORI GAATTCACATTCAGGGTTCTGACCCGGG---p69 GENE---GCCAACGCAGCTGTTAACACT-



1 2 3 4 5 6

Fig. 4.

SUBSTITUTE SHEET

13/13

1 2 3 4 5 6 7 8 9



Fig.5.



SUBSTITUTE SHEET

International Application N

| L CLASS | I. CLASSIFICATION F SUBJECT MATTER (it several classification symbols apply, indicate all) 6 | | | | | | | |
|---|--|--|--------------------------|--|--|--|--|--|
| According | to International Patent Classification (IPC) or to both Na | tional Classification and IPC C 12 N | 1/19, | | | | | |
| IPC ⁵ : | C 12 N 15/81, 15/31 //(C | 12 N 1/19, C 12 R 1 | . 84) | | | | | |
| II. FIELDS | S SEARCHED | | | | | | | |
| Minimum Documentation Searched 7 | | | | | | | | |
| Classification | on System | Classification Symbols | | | | | | |
| IPC ⁵ | C 12 N | | | | | | | |
| | Documentation Searched other to the Extent that such Document | than Minimum Documentation as are included in the Fields Searched * | | | | | | |
| | | 1 | | | | | | |
| | | | | | | | | |
| _IIIDOCU | MENTS CONSIDERED TO BE RELEVANT | | Relevant to Claim No. 12 | | | | | |
| Category * | Citation of Document, 11 with indication, where ap | propriate, of the relevant passages 12 | Research to Cignit sec | | | | | |
| Υ. | Proc. Natl. Acad. Sci 1989, (Wahington, I.G. Charles et a | DC, US), | 1-3,5-10 | | | | | |
| | cloning and charac protective outer r P.69 from Bordete pages 3554-3558 see the whole arts | cterization of nembrane protein lla pertussis", | | | | | | |
| Y | cited in the application Y EP, A, 0341746 (PHILLIPS PETROLEUM CO) 15 November 1989 1-3,5-10 | | | | | | | |
| see claims | | | | | | | | |
| Y | Biotechnology, volume 5, no. 5, May 1987, (New York, NY, US), J.M. Cregg et al.: "High-level expression and efficient assembly of hepatitis B surface antigen in the methylotrophic yeast, Pichia Pastoris", pages 479-485 | | | | | | | |
| "A" doc con "E" eari filin "L" doc whit cital "O" doc oth | * Special categories of cited documenta: 19 "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filling date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosura, use, exhibition or other means "P" document published prior to the international filling date but tart than the priority date and not in conflict with the application but cited to understand the principle or theory underlying the cannot be considered novel or cannot be considered to exhibit the considered to an invention and inventor and inventor and inventor and inventor and the considered to involve an inventive step when the document extend to particular relevance; the claimed invention or other means "P" document published after the international filling date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the considered to exhibit the priority date and not in conflict with the application but cited to understand the principle or theory underlying the claimed invention "X" document of particular relevance; the claimed invention or priority date and not in conflict with the application but cited to understand the principle or theory underlying the claimed invention. "X" document of particular relevance; the claimed invention or priority date and not in conflict with the application but cited to understand the principle or theory underlying the claimed or priority date and not in conflict with the application but cited to understand the principle or theory underlying the claimed or priority date and not in conflict with the application but cited to understand the principle or theory underlying the claimed or priority date and not in conflict with the application but cited to understand the principle o | | | | | | | |
| IV. CERT | IFICATION | | | | | | | |
| Date of the | Actual Completion of the International Search 19th June 1991 | Date of Mailing of this international Se 0 1. 08. 9 | | | | | | |
| | | Signature of Authorized Officer | | | | | | |
| Internation | EUROPEAN PATENT OFFICE | Jague Ho | dul | | | | | |

Form PCT/ISA/210 (second sheet) (January 1985)

| ategory * | LIMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) Citation of Document, 13 with Indication, where appropriate, of the relevant passages Relevant to Claim No. | | | | | |
|-----------|---|----------|--|--|--|--|
| | | | | | | |
| . . | see the whole document | | | | | |
| | cited in the application | ! ! | | | | |
| | | | | | | |
| o,∳,x | Yanak a la a | 1-3,5-10 | | | | |
| | Yeast, volume 6, spec. issue, S428, 1990, 15th International Conference on | | | | | |
| | Yeast genetics and Molecular Biology, M.A. Romanos et al.: "Expression of | | | | | |
| | the bordetella pertussis (whoming | | | | | |
| | cough) P,69 antigen in saccharomyces and pichia", page 2 | | | | | |
| | see abstract | | | | | |
| - | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | , | | | | | |
| | | | | | | |
| | · | | | | | |
| | • | | | | | |
| | | | | | | |
| | · | | | | | |
| | · | | | | | |
| | · | | | | | |
| | • | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| 1 | · | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | • | | | | | |

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

GB 9100487 45887 SA

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 23/07/91

The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| EP-A- 0341746 | 15-11-89 | AU-A- JP-A- | 3473489 2027990 | 16-11-89 30-01-90 | • |
|---------------|----------|----------------|--------------------|---------------------------------------|---|
| | | • | | | |
| | | 5 | | · · · · · · · · · · · · · · · · · · · | |
| | | 4 | | · | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | • | | |
| | | | | | |
| • | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | • | | • | |
| | | | | | |
| | | | | | |

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82